Silencing LncRNA SCAMP1 Inhibits Cell Proliferation in Hepatocellular Carcinoma via Activation of p53 Signaling Pathway

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Research

Keywords: hepatocellular carcinoma, SCAMP1, proliferation, p53 signaling

DOI: https://doi.org/10.21203/rs.3.rs-111303/v1

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Abstract

**Background:** Long noncoding RNA secretory carrier membrane protein 1 (SCAMP1) has been recently reported to be an oncogene in several cancers, including glioma and ovarian cancer. However, its clinical significance and functional role in hepatocellular carcinoma (HCC) remain unknown.

**Methods:** The expression of SCAMP1 was determined in tissue samples and cell lines using quantitative reverse transcription PCR. The clinical significance of SCAMP1 in HCC was evaluated using chi-squared test, Kaplan-Meier survival, as well as univariate and multivariate analysis. Cell proliferation, cell cycle distribution and apoptosis were evaluated using CCK-8 assay, colony formation assay, Flow cytometry analysis. Related protein expression levels were measured by western blot analysis.

**Results:** We found SCAMP1 expression levels were remarkably up-regulated in HCC tissues compared with that in matched adjacent tissues. Increased SCAMP1 expression was significantly correlated with large tumor size, advanced TNM stage and poor survival prognosis in HCC. Knockdown of SCAMP1 significantly inhibited cell proliferation, induced G0/G1 phase arrest and apoptosis in HepG2 and SNU-182 cells. More importantly, knockdown of SCAMP1 downregulated the expression levels of CDK4, Cyclin D1 and Bcl-2, while upregulated the expression levels of p21, p53 and Bax.

**Conclusions:** In summary, our study clarified the oncogenic role of SCAMP1 in HCC, and provided a potential therapeutic target for HCC treatment.

Introduction

As the major subtype of liver cancer, hepatocellular carcinoma (HCC), accounting for almost 80% of all primary liver cancers, is clinically characterized by high prevalence, high mortality rate and drug resistance [1, 2]. At present, significant advances have been made in traditional treatments, including surgical resection, chemotherapy or radiotherapy for HCC, but the five-year survival rate is generally below 50% because of the absence of specific indicator in the early stage [3–5]. Therefore, identification of new therapeutic targets is of great importance for developing effective therapeutic strategy for HCC.

Long non-coding RNAs (lncRNAs) are a group of endogenous transcripts with more than 200 nucleotides in length that exhibit limited or no protein-coding potential [6]. Accumulating evidence has indicated that lncRNAs are involved in almost every aspect of biological processes, including proliferation, cell cycle, apoptosis and invasion [7–9]. It has been reported that aberrantly expressed lncRNAs function as oncogenes or tumor suppressor genes associated with the occurrence and development of various cancers [10, 11]. For example, the expression of SAMMSON was upregulated in HCC tissues, and patients with high levels of SAMMSON had significantly lower overall rate within five years after admission [12]. Upregulation of PDPK2P was clinically associated with a larger tumor embolus, low differentiation, and poor survival, as well as functionally promoted HCC cell proliferation and metastasis [13]. On the contrary, LINC01554 was downregulated in HCC, and its expression was correlated with tumor recurrence, TNM
stage and long-term survival rate of patients [14]. In addition, RUNX1-IT1 [15], EPB41L4A-AS2 [16] and TPTEP1 [17] exert tumor suppressive properties by inhibiting HCC cell growth and proliferation.

Recently, a lncRNA transcript secretory carrier membrane protein 1 (SCAMP1) functions as a carrier and participates in post-Golgi recycling pathway [18], which has been reported to be dysregulated and correlated with the development of various tumors [19, 20]. For instance, Wang et al [21] identified that SCAMP1, as one of the key lncRNAs, is a prognostic biomarker of pancreatic cancer by performing bioinformatic analysis. Functionally, inhibition of SCAMP1 significantly restrained the cell proliferation, migration and invasion, as well as promoted apoptosis in glioma cells [22]. SCAMP1 depletion attenuated cell viability and promoted apoptosis in pediatric renal cell carcinoma under oxidative stress [23]. Similarly, Song et al [24] found that SCAMP1 was highly expressed in ovarian cancer, which promoted ovarian cancer cell invasion and angiogenesis. Nevertheless, the biological function and molecular mechanism of SCAMP1 in HCC have not been fully elucidated. Thus, this study was performed to investigate the expression, clinical significance, biological effects and related molecular mechanisms of SCAMP1 in HCC.

**Materials And Methods**

**Clinical tissue samples**

Tumor tissues and matched adjacent non-cancer tissues were collected from 76 cases of HCC patients who received hepatectomy at Qilu Hospital of Shandong University (Shandong, China) between January 2009 and December 2012 with informed consent from all patients. Before surgery, none of HCC patients received any chemotherapy, radiation therapy or immunotherapy and targeted therapy. The basic demographic and clinicopathological features for HCC patients were described in Table 1. All tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C for further analysis. This study was approved by Institutional Ethics Committee of Qilu Hospital of Shandong University (Shandong, China) based on the Declaration of Helsinki.
Table 1
Association between SCAMP1 expression and clinicopathological characteristics in hepatocellular carcinoma patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n = 76</th>
<th>SCAMP1 expression</th>
<th>P value (chi-square test)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>High (n = 38)</td>
<td>Low (n = 38)</td>
</tr>
<tr>
<td>Age</td>
<td>0.102</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 50</td>
<td>31</td>
<td>12</td>
<td>19</td>
</tr>
<tr>
<td>≥ 50</td>
<td>45</td>
<td>26</td>
<td>19</td>
</tr>
<tr>
<td>Gender</td>
<td>0.139</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>52</td>
<td>23</td>
<td>29</td>
</tr>
<tr>
<td>Female</td>
<td>24</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>HBV</td>
<td>0.442</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>20</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>Present</td>
<td>56</td>
<td>29</td>
<td>26</td>
</tr>
<tr>
<td>Tumor size</td>
<td>0.003*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 5 cm</td>
<td>33</td>
<td>10</td>
<td>23</td>
</tr>
<tr>
<td>≥ 5 cm</td>
<td>43</td>
<td>28</td>
<td>15</td>
</tr>
<tr>
<td>TNM stage</td>
<td>0.006*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II</td>
<td>36</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>III-IV</td>
<td>40</td>
<td>26</td>
<td>14</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>0.243</td>
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<td></td>
</tr>
<tr>
<td>Negative</td>
<td>45</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>Positive</td>
<td>31</td>
<td>18</td>
<td>13</td>
</tr>
</tbody>
</table>

*Statistically significant; Abbreviations: HBV, hepatitis B virus; TNM, tumor-node-metastasis

Cell culture and transfection

A normal transformed human liver epithelial-3 cell line (THLE-3) and two HCC cell lines (HepG2 and SNU-182) were provided by American Type Culture Collection (ATCC, Manassas, VA, USA), which were all cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS, Gibco) and maintained at 37 °C in a humidified atmosphere of 5% CO₂. For cell transfection, two small interfering RNA targeting SCAMP1 (si-SCAMP1#1 and si-SCAMP1#2) and
negative control (si-NC) synthesized by GenePharma (Shanghai, China) were transfected into HepG2 and SNU-182 cells using Lipofectamine 2000 reagent (Invitrogen, California, USA).

**Quantitative reverse transcription PCR**

Total RNA was isolated from tissue samples or cell lines with TRIzol reagent (Life technologies, Carlsbad, CA, USA) and reverse transcription was performed using Prime Script RT reagent Kit (Takara Bio, Japan) following the manufactures’ instructions. Quantitative reverse transcription PCR was carried out using SYBR Green qPCR Master Mix (Takara Bio, Japan) on ABI Prism 7500 Fast Real-Time PCR system (Applied Biosystems, USA) with the primer sequences as follows: SCAMP1 forward: 5′-GATGAGCAGGAGTGGTTGGT-3′ and reverse: 5′-GGTCTCAAGCCTGTATCGTT-3′; GAPDH forward: 5′-TCGACAGTCAGCCGCTTCTTTTT-3′ and 5′-ACCAAATCCCGTTGACTCCGACCTT-3′. Relative expression level of SCAMP1 was normalized to that of endogenous control (GAPDH) using the 2 − ΔΔCt method.

**Cell counting Kit-8 (CCK-8) assay**

After 48 h transfection, cells were seeded into 96-well plates at a density of 3 × 10^3 cells per well and cultured overnight. Next day, a total of 10 µL of CCK-8 solution (Dojindo, Kumamoto, Japan) was added to cells in each well at 0, 24, 48 and 72 h, respectively. After another 2 h incubation at 37 °C, the absorbance was measured at a wavelength of 450 nm by a microplate reader.

**Colony formation assay**

After 48 h transfection, cells were seeded into six-well plates at a density of 500 cells per well and cultured for consecutive two weeks to form cell colony. Subsequently, cell colonies were washed twice with PBS and fixed with 4% paraformaldehyde for 30 min. After stained with crystal violet for 20 min, the colonies were photographed and counted under a light microscope.

**Flow cytometry assay**

For cell cycle analysis, transfected cells were harvested and fixed with 70% ethanol overnight at 4 °C. After washed with PBS, cells were incubated with 0.5 mL of propidium iodide (PI)/RNase Staining Buffer (BD biosciences, San Jose, CA, USA) for 20 min at room temperature. Cell cycle distribution was analyzed by a FACSCalibur flow cytometer (BD Biosciences). For the analysis of apoptosis, transfected cells were washed with PBS twice and stained with Annexin V-FITC and PI for 15 min at room temperature in darkness. The percentage of early (Annexin V+/PI-) and late (Annexin V+/PI+) apoptosis was quantified using flow cytometry (BD Biosciences).

**Western blot analysis**

Total protein samples were extracted with RIPA lysis buffer (Beyotime, Shanghai, China) and quantified with a BCA protein assay kit (Beyotime). Equal amount of protein samples was separated on 10% SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk and then incubated with primary antibodies against CDK4, Cyclin D1, p21, p53, Bcl-2, Bax and GAPDH (all from Abcam, Cambridge, MA, USA) overnight at
4 °C. After washed with PBS twice, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) for 2 h at room temperature. The protein bands were visualized using ECL reagent (Millipore) with GAPDH as the internal control.

**Statistical analysis**

Experimental data were analyzed by GraphPad Prism 6.0 Software (GraphPad Inc., San Diego, CA, USA) and expressed as mean ± standard deviation (SD) from three biological replicates of each experiment. The associations between SCAMP1 expression and clinicopathological features were studied using chi-square test. Overall survival curves were plotted based on Kaplan-Meier method and estimated by the log-rank test. Cox regression analysis was used to determine the independent factors that influenced survival. Paired t test was used to analyze differences between two types of tissues. One-way ANOVA and Tukey test were used to analyze differences among different cell transfection groups. The values of all p less than 0.05 were considered to be statistically significant level.

**Results**

**SCAMP1 was upregulated in HCC tissues and cell lines**

Using quantitative reverse transcription PCR, we determined the expression level of SCAMP1 in tumor tissues and adjacent non-cancer tissues derived from 76 cases of HCC patients. As shown in Fig. 1A, SCAMP1 expression level was significantly upregulated in HCC tissues compared with adjacent tissues. In addition, we detected the expression level of SCAMP1 in HCC cell lines. Consistently, elevated expression of SCAMP1 was also observed in two HCC cell lines (HepG2 and SNU-182), in comparison with human liver epithelial-3 cell line THLE-3 (Fig. 1B).

**High level of SCAMP1 was associated with poor prognosis of HCC patients**

Based on the expression level of SCAMP1 in HCC tissues, we next analyzed the clinical significance of SCAMP1 in HCC. At first, 76 cases of HCC patients were divided into two subgroups (high/low SCAMP1 level) using the median of the cohort as a cut-off value. The correlation analysis between SCAMP1 expression and clinicopathological features of HCC patients was displayed in Table 1, which indicated that high expression of SCAMP1 was positively correlated with large tumor size (p = 0.003) and advanced TNM stage (p = 0.006). Through survival analysis, it was found that patients with the high expression of SCAMP1 had a shorter overall survival time than those with low SCAMP1 expression (Fig. 2, p = 0.0168). Furthermore, the results from Cox regression analysis (Table 2) demonstrated that SCAMP1 expression was an independent prognostic factor for HCC patient survival (HR = 1.006, p = 0.018), in addition to tumor size (HR = 2.015, p = 0.026).
Table 2
Cox regression analysis of different prognostic factors in human hepatocellular carcinoma patients

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td>Age</td>
<td>1.356 (0.921–2.120)</td>
<td>0.116</td>
</tr>
<tr>
<td>Gender</td>
<td>1.256 (0.842–1.895)</td>
<td>0.243</td>
</tr>
<tr>
<td>HBV</td>
<td>2.152 (1.456–2.865)</td>
<td>0.085</td>
</tr>
<tr>
<td>Tumor size</td>
<td>1.562 (0.989–3.285)</td>
<td>0.005*</td>
</tr>
<tr>
<td>TNM stage</td>
<td>1.325 (0.903–2.062)</td>
<td>0.032*</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>2.845 (1.785–3.215)</td>
<td>0.058</td>
</tr>
<tr>
<td>SCAMP1 expression</td>
<td>1.485 (0.985–1.995)</td>
<td>0.003*</td>
</tr>
</tbody>
</table>

**Note:** *Statistically significant; Abbreviations: HBV, hepatitis B virus; TNM, tumor-node-metastasis; HR: hazard ratio; CI: confidence interval; NA, not analyzed*

SCAMP1 knockdown suppressed cell proliferation, induced G0/G1 phase arrest and apoptosis in HCC cells

The increased expression of SCAMP1 was correlated with large tumor size led us to hypothesize that SCAMP1 might promote the HCC cell proliferation in vitro. To this end, SCAMP1 expression was stably depleted in HepG1 and SNU-182 cells with different specific siRNAs. The knockdown efficiency was verified using quantitative reverse transcription PCR (Fig. 3A). The results of CCK-8 assay showed that SCAMP1 knockdown significantly inhibited the proliferation of HepG1 (Fig. 3B) and SNU-182 (Fig. 3C) cells. We observed that si-SCAMP1#1 transfection showed stronger suppressive effects on SCAMP1 expression and cell growth and thus selected it for the subsequent experiments. Next, we further confirmed the suppressive effects of SCAMP1 knockdown on cell proliferation by the results of colony formation assay (Fig. 3D). Considering the close relationship between cell proliferation and cell cycle progression/apoptosis status, we next performed flow cytometry analysis to investigate the effects of SCAMP1 knockdown on HCC cell cycle distribution and apoptosis. As depicted in Fig. 4A, the percentage of cells at G0/G1 phase was significantly increased, while cells at S and G2/M phase were accordingly decreased in si-SCAMP1#1 transfection group compared with si-NC group in both HepG2 and SNU-182 cells. Similarly, SCAMP1 knockdown remarkably promoted cell apoptosis, including early and late apoptosis in both HepG2 and SNU-182 cells (Fig. 4B).

SCAMP1 knockdown activated p53 signaling pathway in HCC cells
To clarify the molecular mechanisms underlying SCAMP1 knockdown regulating HCC cell proliferation, G1/S transition and apoptosis, we analyzed the expression of CDK4/Cyclin D1, associated with G1/S transition and p53 target genes, including p21, p53, Bcl-2 and Bax using western blot analysis. Our results showed that knockdown of SCAMP1 obviously downregulated the expression levels of CDK4, Cyclin D1 and anti-apoptotic Bcl-2, while upregulated the expression levels of p21, p53 and pro-apoptotic Bax in both HepG2 (Fig. 5A) and SNU-182 (Fig. 5B) cells. Taken together, these results indicate that SCAMP1 depletion-mediated inhibition of HCC cell proliferation was associated with the activation of p53 signaling pathway.

**Discussion**

This study focused on the expression level and role of SCAMP1 in HCC. Using quantitative reverse transcription PCR, we first showed that SCAMP1 was highly expressed in HCC tissues and cell lines, and SCAMP1 expression was correlated with tumor size, TNM stage and poor prognosis in HCC patients. Consistent with the upregulation of SCAMP1 in HCC, several studies have reported that the expression of SCAMP1 was increased in ovarian cancer [24], glioma [22] and pediatric renal cell carcinoma [23]. In clinical level, Song et al [24] utilized TCGA database to found ovarian cancer patients with high SCAMP1 level had poorer overall survival rates than those with low SCAMP1. The expression level of SCAMP1 was positively correlated with the pathological grades of glioma and higher SCAMP1 expression indicated poorer overall survival [22]. These results indicated that SCAMP1 might be a potential biomarker in clinical application.

Our *in vitro* experiments showed that SCAMP1 knockdown suppressed cell proliferation, induced cell cycle G0/G1 phase arrest and promoted apoptosis in two HCC cell lines (HepG2 and SNU-182), which suggested that SCAMP1 can be used as an oncogene to promote HCC cell proliferation. In fact, SCAMP1 also plays oncogenic role in other tumors. For instance, inhibition of SCAMP1 significantly restrained the cell proliferation, migration and invasion, as well as promoted apoptosis in glioma [22]. SCAMP1 depletion attenuated cell viability and promoted apoptosis under H$_2$O$_2$ treatment in pediatric renal cell carcinoma [23]. Downregulation of SCAMP1 suppressed the pancreatic and gallbladder cancer proliferation, migration, and invasion [25].

In terms of molecular mechanism, our data further demonstrated that SCAMP1 knockdown reduced the expression of CDK4, Cyclin D1 and Bcl-2, but promoted the expression of p21, p53 and Bax in both HepG2 and SNU-182 cells. Three protein families, including cyclin-dependent kinases (CDKs), Cyclins and CDK inhibitors (CDKIs) regulate the progress of the cell cycle in cancer cells, of which CDK4/Cyclin D1 and p21 are the key regulators of G1/S transition [26, 27]. Here, SCAMP1 knockdown induced cell cycle G0/G1 phase arrest, which might be correlated with decreased CDK4/Cyclin D1 and increased p21 expression induced by depletion of SCAMP1. P53, the tumor suppressor gene product, can directly regulate the p21 gene, which encodes a universal inhibitor of CDKs, to inhibit the cell cycle progression [28]. In addition to p21, Bcl-2 and Bax are the downstream target genes of p53. As our best knowledge, the tumor suppressor protein p53 has a critical role in regulation of the Bcl-2 family, including antiapoptotic protein Bcl-2 and
proapoptotic protein Bax [29, 30]. Our findings revealed that SCAMP1 knockdown induced apoptosis via downregulating Bcl-2 and upregulating Bax expression.

In conclusion, our work demonstrated for the first time that SCAMP1 was overexpressed in HCC tissues and predicted poor prognosis. Moreover, SCAMP1 positively regulated HCC cell growth and proliferation via activation of p53 signaling pathway. Our data provide new insight into the oncogenic roles of SCAMP1 in HCC progression, which may become a potential therapeutic target for HCC.

Declarations

Ethical approval and consent to participate

Written informed consent was obtained from all participants and the work was accepted by the Institutional Ethics Committee of Qilu Hospital of Shandong University (Shandong, China) based on the Declaration of Helsinki.

Authors’ contributions

SXY designed this research. MXC carried out most experiments in this work and drafted this manuscript. LCX performed statistical analysis. ZK and XY help to draft the manuscript. All authors read and approved the final manuscript.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Funding

Not applicable.

Conflict of interest

The authors declare that they have no conflict of interest.

References


